Human Immunodeficiency Virus Type 1 mRNA Expression in Peripheral Blood Cells Predicts Disease Progression Independently of the Numbers of CD4 + Lymphocytes

K Saksela, C Stevens, P Rubinstein, and D Baltimore

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Human immunodeficiency virus type 1 mRNA expression in peripheral blood cells predicts disease progression independently of the numbers of CD4+ lymphocytes

(acquired immunodeficiency syndrome/polymerase chain reaction/3'-azido-3'-deoxythymidine)

KALLE SAKSELA*, CLADD STEVENS†, PABLO RUBINSTEIN†, AND DAVID BALTIMORE*‡

*The Rockefeller University, 1230 York Avenue, New York, NY 10021; and †The New York Blood Center, 310 East 67th Street, New York, NY 10021

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ABSTRACT **To address the significance of human immunodeficiency virus (HIV) replication in peripheral blood mononuclear cells (PBMCs), we have used reverse transcriptase-initiated PCR to measure HIV-1 mRNA expression in PBMC specimens collected from a cohort of HIV-infected individuals during a long-term prospective study. We found dramatic differences in HIV mRNA expression among individuals with very similar clinical and laboratory indices, and this variation strongly correlated with the future course of the disease. No evidence of viral replication was detected in PBMCs from asymptomatic individuals who, thereafter, had normal levels of CD4+ cells for at least 5 years. Irrespective of whether the CD4+ cell numbers were normal at the time of sampling, abundant expression of HIV-1 mRNA in PBMCs predicted accelerated disease progression within the next 2 years. Thus, independently of what may be the rate of HIV replication in other viral reservoirs, such as lymphatic tissue, the amount of HIV mRNA in PBMCs appears to reflect the subsequent development of HIV disease. We have also used the reverse transcriptase-initiated PCR assay to demonstrate a transient response to 3'-azido-3'-deoxythymidine treatment. Determination of HIV-1 mRNA expression in the PBMCs of infected individuals could, therefore, have significant clinical utility as a prognostic indicator and as a means to guiding and monitoring antiviral therapies.**

The pathogenesis of human immunodeficiency virus (HIV) type 1 infection is characterized by a variable but often prolonged asymptomatic period after the acute viremic phase (1). Previous work has established a correlation between HIV disease progression and increasing amounts of infectious virus, viral antigens, and virus-specific nucleic acids (2–12). However, HIV mRNA, indicative of viral replication, has been demonstrated in cells at all stages of the disease, and it has been suggested that true microbiological latency at the cellular level may, therefore, not exist (8, 12). In particular, active viral replication may occur in the lymphatic tissue throughout the clinically latent period (9, 13–15).

Previous studies examining HIV replication in peripheral blood mononuclear cells (PBMCs) of infected individuals have, however, not addressed the critical issue of whether the differences in HIV mRNA levels correlate with the subsequent course of the disease. By examining HIV mRNA expression in serial cryopreserved PBMC samples from clinically well-characterized HIV-infected individuals collected over a 7-year period, we found that the rate of viral replication in PBMCs correlates well with the subsequent clinical course of the infection. We also examined the replication of HIV in PBMCs during antiretroviral therapy and found a significant but transient decrease in HIV-1 mRNA expression when 3'-azido-3'-deoxythymidine (AZT) therapy was initiated during the study.

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MATERIALS AND METHODS

Cryopreserved Ficoll/Hypaque-purified PBMC samples were thawed, washed with phosphate-buffered saline solution, and subjected to RNA extraction using the guanidium isothiocyanate/acid phenol method (16). In addition to information in Fig. 1, a detailed description of the reverse transcriptase-initiated PCR (RT–PCR) and the sequences of the primers has been provided (17). After gel electrophoresis and autoradiography, the intensities of the multiply-spliced (MS) HIV and unspliced (US) HIV signals were compared with those of the control RNAs analyzed in parallel to estimate the approximate amounts of HIV-specific mRNAs present in the patient PBMC samples.

RESULTS

To examine HIV replication in PBMC samples, we developed RT–PCR assays specific for US and MS HIV mRNA as well as cellular β-actin mRNA (Fig. 1*A*). US HIV RNA gives rise to Gag–Pol polyprotein and is also packaged in virions as genomic HIV RNA. MS HIV mRNAs encode regulatory HIV proteins such as Tat and Rev and are not present in virions in significant amounts (K.S. and D.B., unpublished data), thus representing a good measure of ongoing viral replication *in situ*. The RT–PCR of PBMC RNA samples was initiated by a step of random-primed cDNA synthesis followed by specific amplification of diagnostic DNA fragments in the presence of a radiolabeled nucleotide. The PCR products were then analyzed by gel electrophoresis and visualized by autoradiography. To estimate the number of HIV mRNA molecules in the patient samples and to confirm their quantitative amplification after reverse transcription, we prepared control samples by serially diluting known amounts of corresponding *in vitro*-transcribed HIV RNAs into a constant amount of PBMC RNA prepared from an HIV-negative donor. Fig. 1*B* shows a typical result of an RT–PCR analysis of these control RNAs. To control for the amount and integrity of the RNAs prepared from different PBMC samples, as well as the uniform efficiency of their reverse transcription, a β-actin mRNA-specific fragment was also amplified from every cDNA preparation. Variation in the reverse transcription step between the various gene-specific PCR assays was minimized since our assay was based on random-primed reverse transcription, thus allowing the US

Abbreviations: HIV, human immunodeficiency virus; RT–PCR, reverse transcriptase-initiated PCR; PBMC, peripheral blood mononuclear cell; AZT, 3'-azido-3'-deoxythymidine (zidovudine); US, unspliced; MS, multiply spliced; NYBC, New York Blood Center.

‡To whom reprint requests should be addressed.

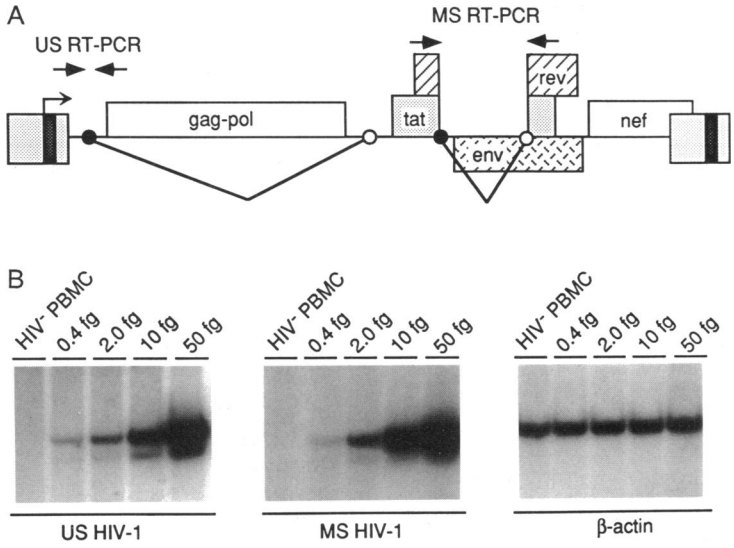

FIG. 1. HIV-1 RT–PCR assay. (*A*) Schematic outline of HIV-1 structure illustrating the relevant viral genes and features of HIV-1 RNA mRNA splicing. The arrows indicate the location of the PCR primers chosen based on maximal HIV-1 sequence conservation and empirical evaluation for optimal performance in RT–PCR. (*B*) RT–PCR analysis of *in vitro*-transcribed HIV-1 RNAs diluted in indicated amounts into a constant amount (1 μg) of total PBMC RNA from a HIV-negative donor. One femtogram (fg) of each control HIV RNA corresponds approximately to 4×10^3 copies of these molecules. A uniform efficiency of reverse transcription among the cDNA reactions is indicated by the comparable intensities of the PBMC-derived β-actin mRNA signals.

HIV, MS HIV, and β-actin mRNAs to be amplified from a single cDNA preparation.

This assay was used to quantitate US and MS HIV mRNA in serial cryopreserved PBMC samples collected from individuals involved in a long-term prospective study at the New York Blood Center (NYBC). The NYBC cohort includes 362 HIV-1-seropositive gay men who entered the study in 1984, 148 of whom had been followed since the late 1970s as a part of a hepatitis B study, allowing timing of seroconversion in 41 patients. Among these 362 men, 224 developed a disease meeting the CDC 1992 criteria for AIDS. Of the remaining, 79 men had evidence of immune deficiency (<500 $CD4^+$ cells per mm^3) but did not develop AIDS, and 59 retained normal $CD4^+$ cell numbers throughout the study.

The present study was prompted by our initial examination of a small number of PBMC samples from individuals who experienced different clinical courses of HIV infection. It revealed abundant HIV mRNA expression in samples from patients with an advanced disease, whereas a large variation was observed in samples from asymptomatic individuals (unpublished data). To address the significance of this variation, 18 individuals were selected from the NYBC cohort, all of whom were initially asymptomatic and had very similar clinical and laboratory indices but whose future course of disease during the subsequent years of follow-up fell into two contrasting patterns (Table 1 and Fig. 2*A*). All 18 individuals included in the study continued to have normal numbers of $CD4^+$ cells during the first 3 years of the follow-up at NYBC. Thereafter 7 of them (patients A–G, group I) developed $CD4^+$ lymphocytopenia and AIDS, whereas the other 11 (patients H–R, group II) continued to have normal $CD4^+$ cell levels and were still asymptomatic when the study was discontinued in 1991.

The first samples from each individual, from which their HIV gene expression pattern was established, were always analyzed in a blinded manner, such that the clinical data were disclosed only after the RT–PCR results were documented. Additional samples from each individual were then examined to define in more detail the temporal aspects of HIV mRNA expression over the years. Fig. 2*B* shows the results from RT–PCR assays of representative PBMC specimens from the 14 individuals included in Fig. 2*A*, while the entire data base of RT–PCR results is summarized in Table 1. These data reveal a strong correlation between HIV replication in PBMCs and the future clinical course of HIV infection. None of the samples from individuals who continued to have normal numbers of $CD4^+$ cells during the subsequent 5 years (patients H–R) expressed a detectable amount of MS HIV-1 mRNA that would indicate viral replication in these cells (Table 1 and Fig. 2*B*). In two cases (patients K and N), a faint US mRNA but no MS HIV signal, was detectable after long autoradiographic exposures (data not shown). Expression of both US and MS HIV-1 RNA was readily detected in all PBMC specimens collected from individuals whose number of $CD4^+$ cells, thereafter, declined below 500 cells per mm^3 within 2 years of sampling. Abundant expression of either HIV mRNA species (Table 1) appeared predictive of active disease progression and was in every case followed by a significant drop in the number of $CD4^+$ cells during the next 1 or 2 years. Thus, as illustrated in Fig. 2, HIV replication in blood cells does not strictly correlate with the clinical stage of HIV infection. Rather, dramatic differences in HIV mRNA expression levels between individuals in the asymptomatic phase of the infection were observed, and this variation strongly correlated with the clinical course of their disease over the subsequent 1–3 years.

As expected, abundant expression of both US and MS HIV-1 mRNA was observed in most samples from individuals with decreased $CD4^+$ cells or AIDS (Table 1). When patients became extensively $CD4^+$ lymphocytopenic, a relative loss of MS HIV-1 mRNA expression was observed, while expression of US mRNA still remained high (e.g., individual C). Cell culture studies have indicated that MS HIV-1 mRNA is predominantly expressed early after HIV infection (18, 19). Thus, the low levels of MS HIV mRNA in some PBMC samples probably reflects inefficient viral spread due to few remaining susceptible uninfected target cells. Also, some of the intense US HIV RT–PCR signal in such samples may be derived from genomic RNA of virions associated with the PBMCs rather than from US HIV mRNA produced in these cells.

To study the effects of antiretroviral therapy on viral replication in PBMCs and to test the possibility that the US and MS HIV mRNA species would be derived from cells with substantially different turnover rates, we also focused on samples collected shortly before and after the initiation of AZT therapy. For this purpose, in addition to the 18 originally selected individuals included in Table 1, serial samples from 3 other AZT-treated individuals were also analyzed (Fig. 3). When AZT treatment was initiated in the interval between the collection of two PBMC samples, a significant decrease in HIV expression was usually observed (Table 1 and Fig. 3). Little or no effect on HIV mRNA expression was seen in one case, probably because AZT was initiated 4 months before the first post-AZT sample was obtained (Table 1, patient D). Despite continuous antiviral therapy, the favorable effect of AZT was typically lost within 4–6 months after treatment initiation. These results correlate with the limited benefit of AZT in the treatment of HIV infection (20) and suggest that

Table 1. Summary of the RT–PCR analyses of HIV-1 mRNA expression in sequential PBMC samples from 18 HIV-infected individuals during a 7-year follow-up period

ID	Date	CD4+ cells, no.	HIV mRNA US	HIV mRNA MS	ID	Date	CD4+ cells, no.	HIV mRNA US	HIV mRNA MS	ID	Date	CD4+ cells, no.	HIV mRNA US	HIV mRNA MS
Disease progression after an asymptomatic period with normal numbers of CD4+ cells														
A	01/30/85	914	++	+	C	02/27/85	881	+	−	E	08/12/84	998	+	−
	01/19/87	1050	+++	++		06/10/86	713	+	+		03/23/86	678	+	−
	09/20/87	645	++	+		03/11/87	909	+	+		04/26/87	1039	+	++
	01/24/88	972	++	++		07/21/87	755	+	++		01/24/88	725	++	+
	06/15/88	72	+++	++		07/24/88	572	+	+		06/12/88	441	+	+
	10/16/88*	99	++	+		09/25/88	703	+++	++		11/13/88	461	+++	+++
	02/26/89	43	+++	+		11/29/88	152	++	++		06/18/89	345	++++	+++
	11/19/89	24	+++	+		04/09/89*	98	+	+		SC < 3/83			
	SC ≤ 10/84	AZT 10/84				10/01/89	29	++	+	F	09/04/84	728	+	−
B	09/10/84	1691	+	+		05/22/90	2	+++	−		07/27/86	660	+	+
	01/28/85	950	+	++		SC 8–10/84	AZT 01/28/89				11/02/88	598	++	+
	02/18/86	732	+	++	D	08/07/84	1076	+	+		04/16/91	188	+++	+
	01/07/87	793	++	+		04/30/85	1480	+	+		SC ≤ 1979			
	10/26/87	798	++	+		09/01/87	986	+	+	G	12/17/84	1122	+	+
	04/19/88	598	++	+++		05/31/88	920	+++	+++		03/11/86	969	+	+
	07/25/88	263	+++	+++		02/14/89	457	+++	++		07/06/86	665	+	+
	09/06/88	258	+++	+++		07/31/90*	385	+++	++		04/27/87	1199	+	+
	03/13/89*	194	+	+		04/23/91	35	+++	+		10/09/88	610	+	++
	07/24/89	136	++	+		SC ≤ 06/80	AZT 04/01/89				02/19/89	252	+++	+++
	04/16/90	39	++	+							04/09/89	117	++	++
	03/12/91	21	+++	+							11/12/89*	92	++	+
	SC < 4/84	AZT 01/13/89									08/05/90	54	++	+
											SC ≤ 3/38	AZT 09/12/89		
No apparent disease progression during the study														
H	12/17/84	1335	−	−	L	09/11/84	1034	−	−	P	11/05/84	1293	−	−
	02/03/88	997	−	−		06/04/85	1493	−	−		07/31/85	1320	−	−
	02/06/91	1080	−	−		01/29/91	894	+	+		08/06/91	1016	−	−
	SC ≤ 3/84					SC ≤ 3/79					SC 2–5/80			
I	08/06/84	1089	−	−	M	08/12/84	1266	−	−	Q	01/29/85	1458	−	−
	09/09/85	1573	−	−		04/21/85	1287	−	−		03/06/88	1955	−	−
	02/04/91	623	−	−		04/17/91	975	−	−		10/08/91	1418	+	+
	SC ≤ 3/84					SC ≤ 3/79					SC ≤ 5/84			
J	01/29/85	1042	−	−	N	07/31/84	817	+	−	R	06/17/85	759	−	−
	09/23/85	783	−	−		08/27/85	952	+	−		05/07/90	468	−	−
	09/09/91	1137	−	−		08/06/91	1159	ND	ND		04/06/91	607	ND	ND
	SC ≤ 4/84					SC ≤ 4/79					SC 8/84–1/85			
K	10/15/84	830	+	−	O	02/04/85	733	−	−					
	08/07/85	1378	−	−		04/01/86	1173	−	−					
	02/06/91	544	++	+		01/27/91	548	−	−					
	SC ≤ 1979					04/08/92	694	ND	ND					
						SC ≤ 5/89								

US and MS HIV-1 mRNA as well as cellular β-actin mRNAs were measured from each sample as described in Fig. 1. ++++, Amount of HIV mRNA corresponding to $>1 \times 10^5$ copies of *in vitro*-transcribed control RNA molecules per μg of PBMC RNA; +++, 0.2–1 × 10^5; ++, 0.5–2 × 10^4; +, 1–5 × 10^3; −, $<1 \times 10^3$ copies. The date and the CD4+ cell number at the time of sampling are shown. In three patients (patients N, O, and R), an additional CD4+ measurement is shown to illustrate the subsequent course of the disease even if HIV mRNA expression was not determined (ND). SC denotes the approximate time when, or before which (≤), seroconversion occurred. When applicable, the date when AZT treatment was initiated is shown, and the first post-AZT specimen is marked with an asterisk (*). ID, patient identification.

determination of HIV-1 mRNA expression in PBMCs could be useful in directly evaluating the effects of antiretroviral therapies and in monitoring the subsequent emergence of drug-resistant virus replication.

The decreased HIV-1 expression after the initiation of AZT therapy also suggests that both US and MS HIV mRNA species are derived from cells with a relatively rapid turnover rate. In contrast to a previous report (21), we found no evidence of latently infected cells expressing abundant MS HIV-1 mRNA but no US HIV-1 mRNA. Rather, any detectable MS HIV mRNA in PBMCs appeared to be incompatible with a long-term (5 years) maintenance of normal levels of CD4+ cells. The absence of MS HIV mRNA, however, did not always indicate that an individual would remain progression-free, because early samples from certain individuals who later experienced disease progression were also found to be negative (patients C, E, and F). Also, readily detectable expression of both US and MS HIV mRNA was observed in recent samples from 3 of the 11 long-term asymptomatic individuals (patients K, L, and Q), suggesting eventual activation of their disease. Nevertheless, lack of MS HIV mRNA correlated in all cases with a benign proximal course of the

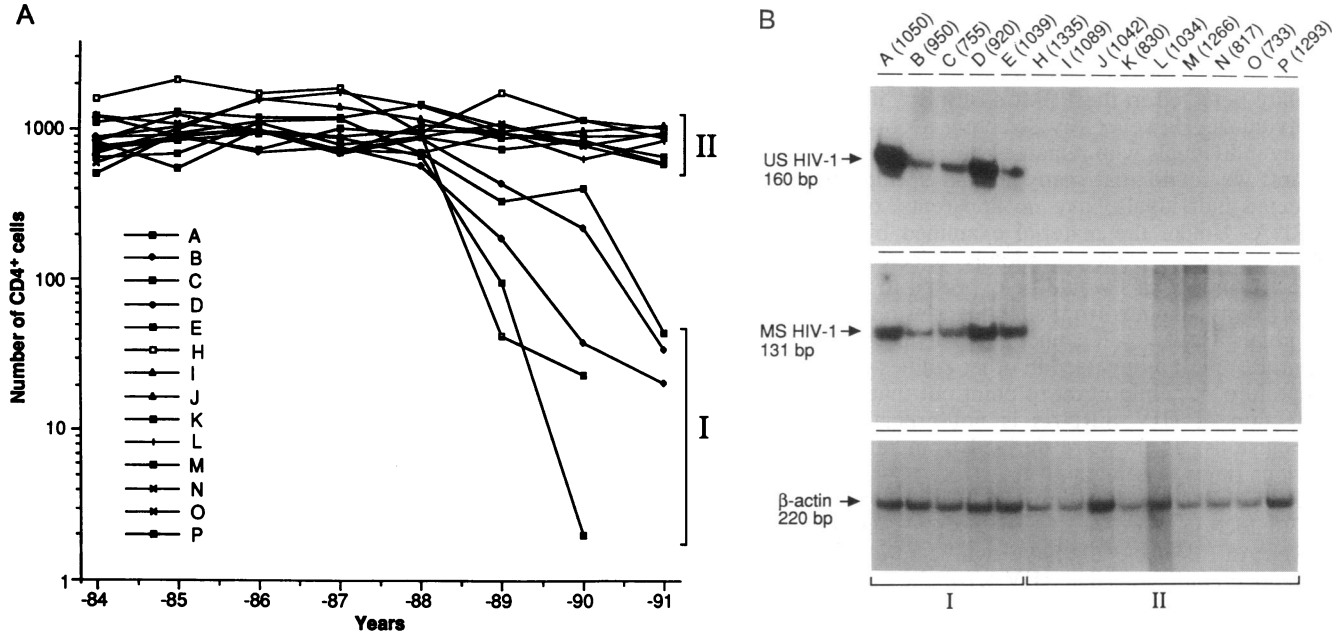

FIG. 2. (*A*) CD4+ cell measurements of 14 individuals during a 7-year follow-up shown on a semilogarithmic scale. The alphabetical codes correspond to those in Table 1. (*B*) RT–PCR analysis of US HIV-1, MS HIV-1 mRNA, and cellular β-actin mRNA in PBMC samples from the 14 individuals included in *A*. Each sample was collected at a time when CD4+ cell numbers were normal, as indicated in the parentheses above the corresponding lanes of the gel.

disease: the time between the last sample negative for MS HIV-1 mRNA and the first CD4+ measurement <500 cells per mm³ was never <2 years and often much longer.

DISCUSSION

The present data show that increased expression of HIV mRNA in PBMCs substantially precedes clinically defined progression of the disease. To our knowledge, longitudinal study addressing the rate of HIV replication in PBMCs has not been previously reported, but results from two studies quantitating viral load during HIV infection are available (4, 11). Both of these studies reported a correlation between viral load and the current disease status but suggested that an increase in viral load could also precede clinical progression of the disease. Viral load in an infected individual is determined by the opposing effects of viral replication and removal of productively infected cells and cell-free HIV. Thus, active HIV replication could occur for long times without an accompanying increase in the overall viral load.

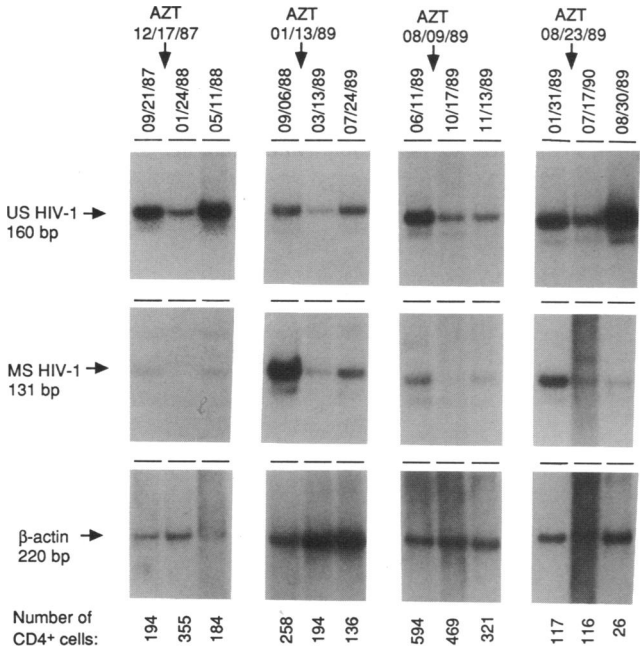

FIG. 3. HIV-1 mRNA expression in sequential PBMC samples from four patients obtained before and after initiation of AZT therapy. As indicated, AZT treatment was started between the collection of the first and the second sample. The dates of sampling and the corresponding measurements of CD4+ cells are also shown. The second panel from the left corresponds to patient B in Table 1.

Our results define a distinct period late in the asymptomatic phase of HIV infection that is characterized by active viral replication in the blood cells in the absence of apparent signs of the immune system destruction. Identification of such a phase of subclinical disease progression may help to characterize the pathogenic process by which HIV infection leads to immunodeficiency and also could have important therapeutic implications.

The underlying causes that activate HIV replication in PBMCs remain unclear. Also, whether the low (undetectable) level of viral mRNA expression observed in the blood cells of some long-term asymptomatic individuals is due more to a particularly effective anti-HIV immune response than to a lower replicative potential of the virus in these individuals remains an important question. The relation of the present findings to the recently recognized active infection in the lymphatic tissues of asymptomatic individuals with little viral replication in their PBMCs (9) is yet another issue that needs to be addressed. It has been suggested that active viral replication in PBMCs would indicate that significant damage in the lymphatic tissues has already occurred (1).

In contrast to the conclusions of an earlier study (21), we found no correlation between a specific HIV mRNA splicing pattern and clinical latency of HIV infection. Exchange of materials with the investigators of that study has revealed that this is due to differences in the performance of the two RT–PCR techniques, particularly the higher sensitivity of the current assay for US HIV-1 RNA (data not shown). Thus, we do not presently believe that long-lived latently infected cells expressing some (MS) but not all (US) HIV mRNA species

represent a significant viral reservoir in asymptomatic HIV-infected individuals.

Previous studies (3, 8, 9) suggested that viral mRNA can be readily detected in the PBMCs of most, if not all, infected individuals. However, by selecting a population including many individuals who remained progression-free for several years, we found that many PBMC specimens from HIV-infected individuals have no apparent expression of HIV mRNA. Unlike the material examined by us, a panel of PBMC samples from a randomly selected group of HIV-infected individuals would be expected to include few relatively early samples from such long-term asymptomatic individuals. However, it cannot be ruled out that the sensitivity of an RT–PCR assay used in other studies could be greater. Therefore, it is important to point out that the threshold of detection of HIV mRNAs is not crucial to the present conclusions, as they are derived from ratios of signals at various times of sampling.

Besides their implications for the pathogenic process of HIV infection, our data also suggest that quantitation of HIV mRNA in PBMCs could have significant clinical utility as a prognostic indicator. The HIV RT–PCR assay was found to reliably distinguish individuals who have otherwise identical laboratory and clinical indices but who will subsequently experience contrasting clinical courses of infection. Based on our data, it is safe to conclude that, independently of the current $CD4^+$ cell numbers, the prognosis for the next 3 years of an individual with undetectable levels of PBMC HIV mRNA differs dramatically from that of an individual showing abundant HIV mRNA expression. We believe that such prognostic information would already be clinically valuable, for example, in examining individuals involved in trials testing antiretroviral therapies, and perhaps in the future, in optimal targeting of such therapies. To establish the value of this approach as a widely used screening test, additional work needs to be done.

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